Characterization of Fish-Skin Gelatin Gels and Films Containing the Antimicrobial Enzyme Lysozyme

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ABSTRACT: Fish skins are rich in collagen and can be used to produce food-grade gelatin. Films cast from fish-skin gelatins are stable at room temperature and can act as a barrier when applied to foods. Lysozyme is a food-safe, antimicrobial enzyme that can also produce gels and films. When cold-water, fish-skin gelatin is enhanced with lysozyme, the resulting film has antimicrobial properties. The objective of this study was to characterize the effect on strength and barrier properties of lysozyme-enhanced fish-skin gelatin gels and films, and evaluate their activity against potential spoilage bacteria. Solutions containing 6.67% fish-skin gelatin were formulated to contain varying levels of hen-egg-white lysozyme. Gels were evaluated for strength, clarity, and viscoelastic properties. Films were evaluated for water activity, water vapor permeability, and antimicrobial barrier capabilities. Fish-skin gels containing 0.1% and 0.01% lysozyme had pH (4.8) and gelling-temperatures (2.1 °C) similar to lysozyme-free fish-skin gelatin controls. However, gel strength decreased (up to 20%). Turbidities of gels, with or without lysozyme, were comparable at all concentrations. Films cast with gelatin containing lysozyme demonstrated similar water vapor permeabilities and water activities. Lysozyme was still detectable in most fish gelatin films. More antimicrobial activity was retained in films cast with higher lysozyme concentrations and in films where lysozyme was added after the gelatin had been initially heated. These results suggest that fish-skin gelatin gels and films, when formulated with lysozyme, may provide a unique, functional barrier to increase the shelf life of food products.

Keywords: antimicrobial edible films, fish, gelatin, gels, lysozyme

Introduction

Gelatin is derived from the thermal degradation of collagen. It is traditionally produced through hydrolysis of bones and skin from cattle and pigs, which can create problems for people with kosher and halal dietary restrictions, as well as pesco-vegetarians. Gelatin produced from fish provides a convenient substitute. Fish skins are rich in collagen and are already being used to produce food-grade gelatin. However, fish collagens, especially from the skins of cold-water fish, have fewer proline and hydroxyproline residues (Gómez-Guillén and others 2002). The resulting gelatin products are capable of less hydrogen-bonding and subsequently have lower gelling temperatures and less gel strength than their mammalian-derived counterparts (Ledward 1986; Leuenberger 1991). This suggests that fish-skin gelatins can offer unique functional properties that differ from commercially prepared mammalian gelatins.

Production of gelatin is an excellent use for fish skins that would otherwise be discarded or made into fish meal by Alaska's fish processing industry. Currently, large volumes of food-grade skins are mechanically separated when boneless fillets are produced from Alaska pollock (*Theragra chalcogramma*), Pacific cod (*Gadus macrocephalus*), and other species. These skins can provide an economical source of raw material for the production of fish-skin gelatin for food and pharmaceutical use. However, as more applications are found for fish-skin gelatin, safety issues may become a concern, since these gelatins can provide a growth medium for bacteria if contamination occurs during manufacturing. Bacterial contamination of gelatin is a well-documented problem. The presence of *Staphylococcus*, *Salmonella*, *Enterococcus*, *Streptococcus*, *Pseudomonas*, and *Yersinia* in gelatins can be attributed to postprocessing contamination (De Clerck and De Vos 2002). However, *Salmonella*, *Staphylococcus*, and sporeformers such as *Bacillus* (De Clerck and others 2004) were also shown to survive the high temperatures and pH extremes of gelatin processing.

Lysozyme is an antimicrobial peptide that is effective against Gram-positive (and sometimes Gram-negative) bacteria (Masschalck and Michiels 2003). It is found in all mammals, birds, and fishes (Magnadóttir 2006). Hen-egg-white lysozyme is capable of forming a gel as well as a strong, transparent film (Johnson and Zabik 1981). The purpose of this study was to characterize the effect on strength and barrier properties of lysozyme-enhanced fish-skin gelatin gels and films, and to evaluate their efficacy as antimicrobial barriers to contamination.

Materials and Methods

Gelatin

Gelatin solutions (6.67% w/w) were prepared from high molecular weight cold-water fish-skin gelatin (Norland Products, Inc., Cranbury, N.J., U.S.A.) and pollock-skin gelatin extracted in our laboratory. Pollock (*T. chalcogramma*) skins obtained from a commercial fish processing plant in Alaska were processed into gelatin using the following procedure: skins were washed in ice water containing 0.8 *N* NaCl solution (to remove flesh and other impurities); the cleaned skins were then washed (4 °C) with 0.2 *N* NaOH, followed by 0.2 *N* H_2SO_4, and then 0.7% citric acid, so that only translucent collagen

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remained; skins were heated (45 °C, 15 h) to hydrolyze the collagen, and then the solution was filtered under vacuum using Whatman No. 4 filter paper; the clear filtrate was air-dried in a convection oven at 45 °C until moisture was less than 15%; final yield was estimated at 13% (weight of dried gelatin/weight of thawed fish skin). Gelatin solutions were formulated to contain levels varying from 0.001% to 0.1% (0.01 mg/mL to 1.0 mg/mL) of hen-egg-white lysozyme (Sigma, St. Louis, Mo., U.S.A.).

Gelatin film casting

Gelatin solutions (6.66% w/w) were heated (40 °C, 20 min) with concentrations of lysozyme ranging from 0.01 mg/mL to 5.0 mg/mL, and then poured onto a flat Mylar surface. Films were spread to uniform thickness using a stainless steel drawdown bar with a 2.0-mm gap, and allowed to dry overnight at ambient conditions. Film thicknesses were measured with a micrometer IP 65 (Mitutoyo Manufacturing, Tokyo, Japan) to the nearest 0.00254 mm (0.0001 in.) at 5 random positions around the film. The mean value was used to calculate water vapor permeability.

Gel clarity, pH, and A_w

Gel clarity was recorded as transmittance of 6.67% (w/v) gelatin solutions with or without lysozyme at 600 nm in a spectrophotometer at 21 °C. The pH of each gelatin and gelatin-lysozyme solution was measured using a Beckman Mod 390 pH meter (Beckman Instruments Inc., Fullerton, Calif., U.S.A.). Water activities were measured with an AquaLab CX (Decagon Devices, Inc., Pullman, Wash., U.S.A.).

Gel strength

Dry gelatin was dissolved in distilled water at a concentration of 6.67% (w/v). Some gel solutions were heated (60 °C, 1 h) with lysozyme (0.01% or 0.1%), and others were combined with lysozyme after heating to minimize the possibility of enzyme denaturation through exposure to high temperatures. Gelatin gel solutions were degassed, then 25 g samples were poured into 30 mL beakers and allowed to mature (7 °C, 18 h) to attain maximum stability. Dimensions of the samples were 3.3 cm^2 and 6 cm height. Gel strength was determined at 2 °C using an Instron Model 55R4502 Universal Testing Machine (Instron Corp., Canton, Mass., U.S.A.) with a 100-N load cell, cross-head speed of 10 mm/min, equipped with a 1.27-cm dia flat-faced cylindrical stainless steel plunger. Maximum force (in N) was taken when the plunger penetrated 4 mm into the gelatin gels, with 4 replications for each treatment. The firmness of each gel was recorded at the first peak.

Gel set temperature

Viscoelastic studies were conducted using a Brookfield DV-III rheometer (Brookfield Engineering Lab, Middleboro, Mass., U.S.A.). A small sample adapter, equipped with a size 21 cylindrical spindle set for 10 rpm, was used to measure the gel set point of the gelatin. Room temperature samples (8.5 g) were cooled from 25 °C to 2 °C at a rate of 4 °C/min until the gel set point was reached.

Water vapor permeability

Water vapor permeability of the gelatin films was determined by estimating the percent relative humidity (RH) at the film underside (McHugh and others 1993). Poly methyl methacrylate (Plexiglas™) test cells (50.8 mm dia) were filled with 6 mL of water and a silicone sealant was applied to the top edges. Gelatin films were placed onto the test cells and Plexiglas rings were tightly applied to prevent vapor leaks. Cells were placed into a humidity chamber (25 °C/0% RH) and allowed to equilibrate (2 h). Weights were periodically measured to assess moisture loss. Linear regression analysis of the slope of weight loss plotted against time was used to determine the water vapor transmission rate in g/h. The permeability was calculated as (g mm)/(kPa h m^2) by considering the test cell mouth area, the percent difference in RH from the film's underside, and the mean film thickness (McHugh and others 1993).

SDS polyacrylamide gel electrophoresis

Molecular weight comparisons for gelatin samples were determined using NuPAGE® Novex gels (10% Bis-Tris) with NuPAGE MOPS SDS running buffer (Invitrogen Corporation, Carlsbad, Calif., U.S.A.). A BenchMark Protein Ladder (10 to 220 kD) was selected as a standard to include a molecular weight range from lysozyme (18 kD) to gelatin β-bands (200 kD). Samples were diluted to a protein concentration of 0.5 μg per lane before being denatured with NuPAGE LDS Sample Buffer and reduced with dithiothreitol (70 °C, 10 min). The gels were run (200 V, 50 min) using an XCell SureLock™ electrophoresis unit (Invitrogen Life Technologies, Carlsbad, Calif., U.S.A.), and then stained with Invitrogen SimplyBlue™ according to the procedures provided by the manufacturer.

Microbiological evaluation of gelatin gels and films

The antimicrobial activity of gelatin films containing lysozyme was challenged with *Escherichia coli*, *Bacillus subtilis* (NRRL-B-645), and *Streptococcus cremoris* (NRRL-B-634). This was accomplished by preparing Trypticase Soy Agar media (Becton, Dickinson and Co., Franklin Lakes, N.J., U.S.A.) according to the manufacturers instructions, and then seeding the media with a 0.1% suspension of *E. coli*, *B. subtilis*, or *S. cremoris* before pouring the plates. Bacterial challenge studies were conducted by preparing gelatin gels and films containing different concentrations of lysozyme (ranging from 0.01 mg/mL to 5 mg/mL) and then exposing them to the seeded agar plates. For evaluating gels, small wells (6 mm dia) were created within each agar plate, and then filled with gelatin samples (50 μL). To evaluate films, small disks (6 mm dia) were punched from each film and placed on the seeded agar. All plates were incubated at 35 °C for 24 h. Gels and films that retained their antibacterial activity produced zones of inhibition, which could be used to compare the efficacy of each lysozyme-gelatin formulation.

Statistical evaluation

A one-way analysis of variance procedure was used with Statistica software, release 6 (StatSoft Inc., Tulsa, Okla., U.S.A.). Tests of significance for treatments were all significant ($p < 0.05$). Post hoc analysis used the Duncan test and the level of significance used was $P < 0.05$. Minimum sample size for experimental analyses was $n = 3$.

Results and Discussion

pH

Fish-skin gelatin gels containing 0.01% and 0.1% lysozyme had a pH similar to the lysozyme-free control of pH 4.8 (Table 1). The pH of a gelatin solution is an important factor for controlling gel strength and solubility. The gel strength of a gelatin is maximum on either side of its isoelectric point ($pH_{iso} = 4.8$; Egelandsdal 1980). This gel-forming ability (measured in units of Bloom) directly affects the cost of the product, with higher prices garnered for higher bloom values. Gelatin has a pH-dependent net electrical charge, which binds small anions and cations (Stainsby 1977). Additives can sometimes shift the charge within a gelatin solution, seriously affecting the gel strength and sometimes resulting in gelatin precipitation. This is not an issue when lysozyme is incorporated as an antimicrobial agent

Table 1 — Characteristics of lysozyme-enhanced fish-skin gelatin

	Gel pH (at 21 °C)	Transmittance (21 °C, 600 nm)	Gel strength (N at 8 °C)	Temperature of gelation (°C)
Control (0% lysozyme)	4.86 (0.01)^{abc}	85.7% (0.84)^{a}	0.733 (0.042)^{a}	2.1 (0.07)^{a}
0.01% Lysozyme (heated 60 °C, 60 min)	4.95 (0.01)^{b}	83.3% (0.14)^{b}	0.683 (0.017)^{ab}	2.1 (<0.01)^{a}
0.1% Lysozyme (heated 60 °C, 60 min)	4.80 (0.02)^{acd}	82.2% (0.19)^{b}	0.613 (0.029)^{ab}	2.1 (<0.01)^{a}
0.01% Lysozyme (not heated)	4.76 (0.05)^{acd}	82.1% (0.13)^{b}	0.626 (0.012)^{ab}	2.1 (<0.01)^{a}
0.1% Lysozyme (not heated)	4.72 (0.04)^{cd}	77.2% (0.13)^{c}	0.589 (0.044)^{b}	2.1 (<0.01)^{a}

Commercial gelatin concentration was 6.67% w/w. Data are presented as Mean Values ± Standard Deviation.
Different letters within a column indicate difference at $p < 0.05$.

into gelatin gels, since the pH values were similar and no precipitation was observed.

Gel clarity

The transmittance through each gelatin solution was measured at 600 nm (Table 1). The samples containing lysozyme appeared visually similar at all concentrations; however, spectrophotometric readings were decreased, indicating reduced clarities when compared to the lysozyme-free control gel. The high values recorded for the unheated 0.1% lysozyme-gel solution likely reflected the presence of light-scattering microaggregates, which formed within the solution as a consequence of not reheating it after the addition of lysozyme. Both color and turbidity of a gelatin gel can be important aesthetic properties, depending on the application for which the gelatin is intended.

Gel strength

Lysozyme-enhanced gelatin gels were tested for strength (Table 1). In all cases, gel strength decreased (up to 20%) when lysozyme was present. At high concentrations, lysozyme by itself produces a firm gel due to its high concentration of disulfide groups and its globular conformation (Johnson and Zabik 1981). However, rather than forming ionic bridges with the negatively charged gelatin to increase gel strength, the small cationic lysozyme molecules produced the opposite effect. There is precedence for expecting additives to improve the functional properties of gelatin. Gel strength, adhesion, foaming, water absorption, and enhanced film-forming characteristics have been previously achieved through modification of commercial gelatins (Taylor and others 2004). However, in this study, it is possible that the lysozyme competed within the system to bind water, leading to reduced gelatin solubility and a lower overall gel strength. The lysozyme may have also blocked the interaction and alignment of critical gel-forming residues, further decreasing the gel strength.

The thermal stability of a gelatin gel depends on the hydroxyproline content of the collagen from which the gelatin was derived (Ledward 1986). Those with low hydroxyproline contents typically have low gel strengths (Gómez-Guillén and others 2002). The strength of a gel is most heavily influenced by its concentration of alpha-chains (95 kD; Fernández-Díaz and others 2003). Gelatin solutions containing lysozyme were compared using SDS polyacrylamide gel electrophoresis (Figure 1). Low concentrations of alpha-chains, accompanied by protein fragments that appear as extraneous bands are indicative of a poor quality gelatin. Freshly prepared pollock-skin gelatin displayed less degradation of alpha-chains than the commercially available fish-skin gelatin chosen for testing (Figure 1). When lysozyme was added to identical concentrations of these gelatins, the lysozyme band was less visible in the lanes containing pollock-skin gelatin, suggesting that it was binding to some component in the gelatin.

Gel set temperature

Gelling temperatures (2.1 °C) were identical for gelatins enhanced with lysozyme and their lysozyme-free controls (Table 1). The concentration of lysozyme incorporated into the gels had no effect on the temperature of gelation, nor did the time of lysozyme addition or the pre- or postheating protocols of gel preparation.

Gelatins from cold-water fish skins have a lower melting point than mammalian gelatins, due to their low concentration of proline and hydroxyproline amino acid residues. This reduces the propensity for intermolecular helix formation (Gómez-Guillén and others 2002). The viscosity in solution is an important parameter when describing the characteristics of a gelatin. The high viscosity of fish-skin gelatin solutions at low temperature, which occurs without the formation of a gel, offers an alternate functionality to food processors.

Water activity of films

Films cast from lysozyme-enhanced gelatin had water activities identical to the lysozyme-free control (0.37) as shown in Table 2. Gelatin that is dried to a very low moisture content may form cross-links, thereby losing solubility, making it difficult to distinguish between free and bound water in gelatin (McCormick-Goodhart 1995). The moisture content of dried gelatin powders can be as high as

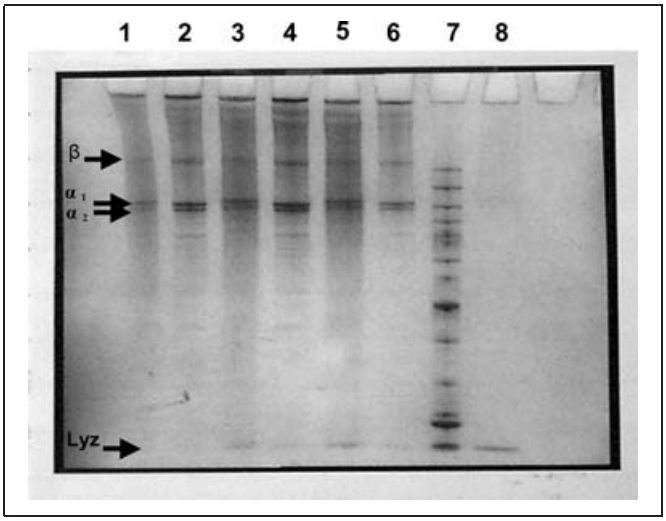

Figure 1 — SDS polyacrylamide gel electrophoresis of a commercially produced fish-skin gelatin and a pollock-skin gelatin, with or without addition of the antimicrobial protein lysozyme. The lanes contained: (1) commercial fish-skin gelatin; (2) pollock-skin gelatin; (3) lysozyme (1 mg/mL) in commercial fish-skin gelatin; (4) lysozyme (1 mg/mL) in pollock-skin gelatin; (5) lysozyme (3 mg/mL) in commercial fish-skin gelatin; (6) lysozyme (3 mg/mL) in pollock-skin gelatin; (7) protein standard; (8) lysozyme standard (1 mg/mL). The gelatin β (200 kD) and α (95 kD) bands have also been labeled.

Table 2—Water vapor permeability of lysozyme-enhanced commercial fish-skin gelatin films

	Water activity (A_w)	Water permeability [(g mm)/(kPa h m^2)]
Control (0% lysozyme)	0.37 (0.01)[a]	0.94 (<0.3)[a]
0.01% Lysozyme (heated 60 °C, 60 min)	0.37 (0.01)[a]	1.19 (0.37)[a]
0.1% Lysozyme (heated 60 °C, 60 min)	0.37 (0.01)[a]	1.20 (0.19)[a]
0.01% Lysozyme (not heated)	0.37 (0.01)[a]	1.00 (0.18)[a]
0.1% Lysozyme (not heated)	0.37 (0.01)[a]	1.04 (<0.3)[a]

Commercial gelatin concentration was 6.67% w/w. Data are presented as Mean Values ± Standard Deviation.
Different letters within a column indicate difference at $p < 0.05$.

16%, although it typically ranges from 10% to 13% (McCormick-Goodhart 1995). Lower moisture levels (6% to 8%) can produce a gelatin powder that is very hygroscopic (McCormick-Goodhart 1995); however, this was not found with any of the gelatin films in this study.

Water vapor permeability of gelatin films

Films prepared from fish-skin gelatins formulated with lysozyme appeared to demonstrate a slight increase in permeability to water vapor, as compared to the control films (Table 2). Water vapor transmissibility rates of the lysozyme-containing films were higher, suggesting a decreased value of the film as a barrier to moisture. Water permeability values were lowest for the control commercial gelatin film and highest for films made with lysozyme heated to 60 °C, although statistically there were no significant differences between treatments ($P > 0.05$). Both water permeability and water transmissibility rates were lowest for films made from gelatin without lysozyme. These results support previously described findings that gel strength decreases with the addition of lysozyme and is most likely due to similar deleterious interactions occurring between lysozyme and gelatin.

Lysozyme activity in gelatin films and gels

Lysozyme-enhanced gelatin films and gels, prepared from commercial fish-skin gelatin, were subjected to bacterial challenge studies to test their resistance to microbial contamination. Three bacteria, *Escherichia coli*, *Bacillus subtilis*, and *Streptococcus cremoris*, were chosen since all are potential contaminants in gelatin (De Clerck and De Vos 2002). As expected, the lysozyme-enhanced gelatin films and gels produced no zones of inhibition on plates seeded with Gram-negative *E. coli* cultures, since lysozyme does not penetrate the lipopolysaccharide layer of Gram-negative bacteria unless the cells are pretreated first (Takada and others 1994). However, gelatin gels and films formulated with lysozyme at concentrations as low as 0.001% (0.01 mg/mL) remained effective against both Gram-positive bacteria tested. Results for *B. subtilis* are shown in Figure 2.

Some bacteria were already present in the dry gelatin powders assembled for this study; however, no colonies survived the process of heating to 60 °C, which was used to make the gels and films. None of the bacteria isolated from the original gelatin stocks demonstrated gelatinase activity, which can affect the quality of the gel. Contaminants that produce gelatinase are capable of breaking down the gelatin matrix, thereby releasing more nutrients and promoting further bacterial growth.

Bacillus species form spores that can be very resistant to heat, chemicals, and desiccation. The complete destruction of spores in gelatin would require severe processing conditions that would negatively impact the technical properties of the gelatin. Discovery of a *Streptococcus* species in gelatin would likely signify postprocessing contamination. The presence of lysozyme as an antimicrobial agent appeared to be effective for controlling these two varieties of Gram-positive bacteria in gelatin films.

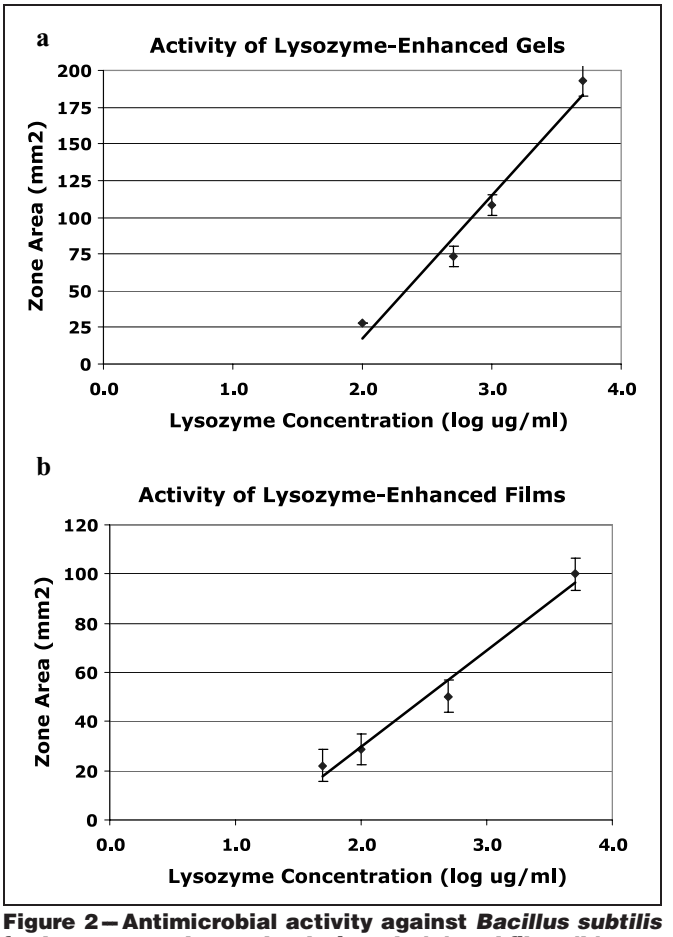

Figure 2—Antimicrobial activity against *Bacillus subtilis* for lysozyme-enhanced gelatin gels (a) and films (b), prepared from commercial gelatin (6.67% w/w)

Conclusion

The presence of lysozyme in fish-skin gelatin had little effect on some gel characteristics (such as pH and the gelation temperature); however, other properties such as gel clarity and gel strength were impacted. Lysozyme-enhanced films showed promise for retaining antibacterial activity and inhibiting bacterial contaminants. These results suggest that fish-skin gelatin gels and films, when formulated with lysozyme, may provide a unique, functional barrier to increase the shelf life of food products.

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